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Research paper

Markedly different immune responses and virus kinetics in littermates infected with porcine circovirus type 2 or porcine parvovirus type 1

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ABSTRACT

Porcine parvovirus type 1 (PPV1) and porcine circovirus type 2 (PCV2) are small single-stranded DNA viruses with high prevalence in the global pig population. The aim of this study was to compare and contrast PCV2 and PPV1 infections in high-health status pigs and to describe PCV2 long-term infection dynamics. Six caesarian-derived colostrum-deprived pigs were randomly divided into two groups and were experimentally infected with PCV2 or PPV1 at 5 weeks of age. All pigs had detectable viremia by day (D) 3 post-infection. Pigs infected with PPV1 had a detectable INF- α response by D3 followed by a high IFN- γ response by D6. The PPV1 pigs developed antibodies against PPV1 by D6 resulting in decreasing virus titers until PPV1 DNA became undetectable from D28 until D42. In contrast, PCV2-infected pigs had no detectable INF- α or IFN- γ response after PCV2 infection. PCV2-infected pigs had no detectable anti-PCV2 humoral response until D49 and had a sustained high level of PCV2 DNA for the duration of the study. While PPV1-infected pigs were clinically normal, PCV2-infected pigs developed severe clinical illness including fatal systemic porcine circovirus associated disease (PCVAD) by D28, fatal enteric PCVAD by D56 and chronic PCVAD manifested as decreased weight gain and periods of diarrhea. Microscopically, all three PCV2-infected pigs had lymphoid lesions consistent with PCVAD and associated with low (chronic disease) to high (acute disease) levels of PCV2 antigen. Under the study conditions, there was a lack of early IFN- γ and INF- α activation followed by a delayed and low humoral immune response and persisting viremia with PCV2 infection. In contrast, PPV1-infected pigs had IFN- γ and INF- α activation and an effective immune response to the PPV1 infection.

1. Introduction

Several small, non-enveloped, single-stranded DNA viruses circulate in pigs including porcine circovirus type 2 (PCV2) and porcine parvovirus type 1 (PPV1). Both of these viruses are highly resistant to inactivation and widespread in pig herds worldwide. While there are several parvovirus species (PPV1 (Dunne et al., 1965), PPV2 (Hijikata et al., 2001), PPV3 (Lau et al., 2008), PPV4 (Cheung et al., 2010), PPV5 (Xiao et al., 2013), PPV6 (Ni et al., 2014), PPV7 (Palinski et al., 2016)), only PPV1 is typically associated with disease. PPV1 was first identified in the 1960s as a cause of reproductive failure in breeding herds (Mengeling et al., 1991) but can also cause cutaneous lesions in growing pigs (Kresse et al., 1985; Lager and Mengeling, 1994). PPV1 has a tropism for actively replicating cells such as fetal myocardiocytes and fetal infection often results in death, which, depending on the stage of pregnancy, can manifest as increased numbers of mummified fetuses,

stillborn or weak born pigs. Vaccines to control PPV1 are routinely used in breeding herds.

PCV2 is associated with several different clinical presentations of pigs referred to as PCV2 associated disease or PCVAD. Systemic disease (Opriessnig et al., 2007) or postweaning multisystemic wasting syndrome (Harding, 2007) in growing pigs is the most common PCVAD. PCV2 also plays a role in the porcine respiratory disease complex (Harms et al., 2002; Kim et al., 2003) and has been associated with enteric disease in grow-finish pigs (Jensen et al., 2006; Opriessnig et al., 2011a). An association with porcine dermatitis and nephropathy syndrome (PDNS) is also suspected (Rosell et al., 1999); however, PDNS may occur in pigs free of PCV2 indicating that several factors can cause this disease syndrome. PCV2 strains can be divided into different genotypes of which PCV2a was the most prevalent genotype before 2000 (Fenaux et al., 2000), followed by a global shift towards PCV2b (Patterson and Opriessnig, 2010) which has since been followed by

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another shift towards PCV2d (Xiao et al., 2015). While differences in pathogenicity between PCV2 genotypes are suspected, experimental comparison of strains revealed that strain differences may be more important than genotype differences (Opriessnig et al., 2008; Opriessnig et al., 2014b). Vaccines to control PCV2 are routinely used in growing pigs and less frequently in the breeding herd.

The aim of this study was to generate anti-PPV1 and anti-PCV2 antiserum over time from high health status pigs; however, unique infection kinetics were observed and thus are described in this manuscript.

2. Materials and methods

2.1. Ethical statement

The experimental protocols were approved by the Iowa State University Institutional Animal Care and Use Committee (Approval number: 1-11-7071-S).

2.2. Animals, housing, experimental design and serum collection

Six 4-week-old, caesarian-derived colostrum-deprived (CDCD) male pigs from one litter were obtained from a commercial source (Struve Labs International, Manning, IA, USA), transported to Iowa State University, and randomly divided into two groups and rooms of three pigs. The aim of the study was to generate antibody positive control serum for PCV2 and PPV1 for future evaluation and standardization of serology assays. The experimental design is summarized in Fig. 1. The experimental endpoint of the study was initially day 49 (D49) as antibody levels to PPV1 and PCV2 are usually high at that time. To obtain serum reflecting seroconversion with low to medium levels of antibodies, blood was collected initially every three days until D15 then on D21 and weekly thereafter. Blood was collected in serum separator tubes (Fisher Scientific, Pittsburgh, PA, USA), centrifuged at $3000 \times g$ for 10 min at 4°C and the serum was stored at -80°C until testing.

2.3. Inoculation

At 5 weeks of age, pigs 121, 124 and 125 in the PCV2 group were inoculated using an infectious PCV2a stock (strain 40895; Fenaux et al., 2003) with a titer of $10^{4.5}$ 50% tissue culture infectious dose (TCID₅₀) per ml intramuscularly (1 ml) and intranasally (1 ml). For the PPV1 inoculation, a frozen tissue stock inoculum (NADC, Ames, Iowa) was used as described (Opriessnig et al., 2011b). In brief, PPV isolate NADL-8 was isolated in fetal porcine kidney cells from a naturally infected pig in 1977 (Mengeling, 1979) and was subsequently passaged in fetuses in the pregnant sow model. Passage 4 of the PPV challenge virus stock was used at an approximate titer of $10^{4.9}$ TCID₅₀ per ml (Opriessnig et al.,

2011b). Each pig in the PPV1 group (pigs 99, 122 and 123) received 1 ml of the PPV1 tissue homogenate intranasally by slowly dripping 0.5 ml into each nostril.

2.4. Clinical observations

All animals were examined daily for signs of illness such as lethargy, respiratory disease, diarrhea, inappetence and lameness.

2.5. Serology

All serum samples were tested for the presence of PCV2 IgG by an *in house* indirect ORF2-based ELISA (Nawagitgul et al., 2002). Samples were considered positive if the sample-to-positive (S/P) ratio was equal to or greater than 0.2 (Nawagitgul et al., 2002). In addition, all samples were tested by a commercial blocking whole PCV2-based anti-PCV2 IgG ELISA (SERELISA® PCV2 Ab Mono Blocking; Zoetis). Sample titers were calculated based on single dilutions using the calculation sheet supplied by the manufacturer. To determine the presence of PPV1 antibodies, all serum samples were tested by a commercial blocking ELISA (Ingezim PPV Compac; R.11.PPV.K3; Ingenasa, Madrid, Spain). This assay is based on recombinant VP2 PPV1 protein. Samples with a blocking percentage $> 30\%$ are considered positive, samples $< 25\%$ are considered negative, and samples with a blocking percentage from 25 to 30% are considered indeterminate and could be positive or negative. Serum samples collected at D21 and D35 were also tested for the presence of PPV1 antibodies by a hemagglutination inhibition (HI) assay as described (Mengeling et al., 1988).

2.6. DNA extraction, detection and quantification amount of PCV2 and PPV1

Total nucleic acids were extracted from all serum samples using the MagMax™ Pathogen RNA Kit (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) on an automated nucleic acid extraction system (Thermo Scientific Kingfisher® Flex, Thermo Fisher Scientific, Pittsburgh, PA, USA) according to the instructions of the manufacturer. Negative and positive controls were included in each run. All DNA extracts were tested for the presence of PCV2 with an ORF1-based real-time PCR assay as described (Opriessnig et al., 2003). In addition, all DNA extracts were tested for the presence of PPV1 by a quantitative PPV1 real-time PCR assay targeting the VP2 region (Opriessnig et al., 2011b). For both assays, samples were considered negative when no signal was observed by a cycle-threshold (C_T) of 40. To further characterize the PCV2 strain present in the pigs, selected PCV2 DNA positive serum samples were tested by a PCV2a/2b/2d differential real-time PCR assay targeting the ORF2 (Opriessnig et al., 2010a; Opriessnig et al., 2014a). Selected PCV2 DNA positive samples were also subjected

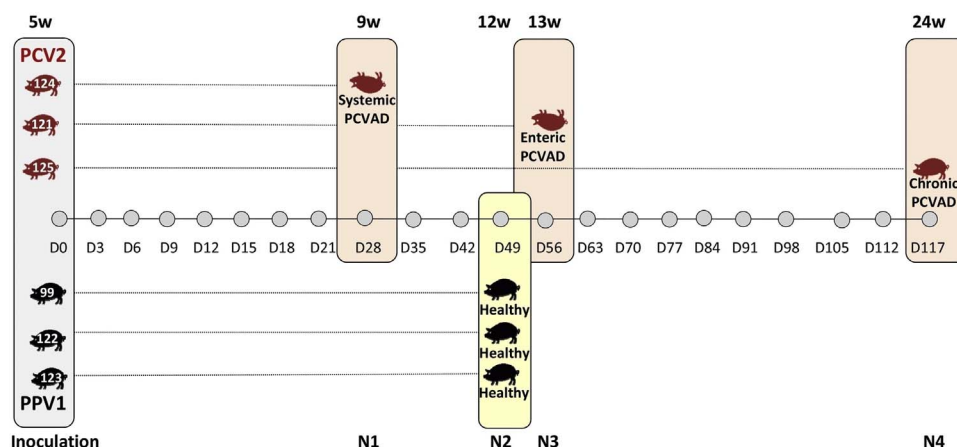


Fig. 1. Experimental design. Blood samples were collected on certain days indicated by grey circles. The age of the pigs in weeks is indicated on top. N = necropsy.

to sequencing of ORF2 (Gerber et al., 2013).

2.7. Cytokine analysis via multiplex fluorescent microsphere immunoassay (FMIA)

Serum samples collected on D0, D3, D6, D9, D12, D15 and D21 were analyzed for IL1- β , IL-8, IFN- α , IL-10, and INF- γ by a 5-plex FMIA (Ladinig et al., 2014; Lawson et al., 2010). Due to reagent availability, the IL-8 standard was replaced by the swine IL-8 recombinant protein (Cat. N. RP0109S-025, KingFisher, Saint Paul, MN, US). All other standards, capture and detection antibodies were as described (Ladinig et al., 2014) and INF- γ was coupled to magnetic beads as described elsewhere (Lawson et al., 2010). Cytokine standards were diluted with phosphate buffered saline (PBS, pH 7.4). Serum samples were tested at a 1:3 dilution in PBS supplemented with 1% bovine serum albumin (BSA) and 0.05% proclin (PBS-B). TNF α levels were estimated using the MILLIPLEX MAP porcine cytokine/chemokine magnetic bead panel (Millipore, Merck KGaA, Darmstadt, Germany) according to the manufacturer's protocol. Coupled microspheres were tested on a MagPix system (Biorad, Austin, Texas, USA) and results were analyzed with the Bio-Plex Manager software version 6.1 (Biorad). All mean fluorescence intensity (MFI) measurements were corrected for their background by subtracting the MFI of the blank control (beads incubated without serum samples) from the MFI for the relevant analyte in each sample.

2.8. Necropsy

Pigs were euthanized when developing clinical disease or on D49 whichever came first (Fig. 1). By D49 the two remaining PCV2 pigs had a delayed antibody response and therefore they were kept longer to allow for seroconversion. Euthanasia was done by intravenous pentobarbital sodium overdose (Fatal Plus[®], Vortech Pharmaceuticals, LTD, Dearborn, MI, USA). Gross lesions were assessed and recorded. Sections of the mediastinal, mesenteric, superficial inguinal, external iliac and tracheobronchial lymph nodes, liver, kidney, spleen, tonsil and lungs were collected during necropsy and fixed in 10% neutral-buffered formalin and routinely processed for histopathology.

2.9. Histopathology and immunohistochemistry

Microscopic lesions in PCV2 pigs were evaluated by a veterinary pathologist blinded to the treatment groups. Lung tissue sections were scored for the presence and severity of interstitial pneumonia, with scores ranging from 0 (normal) to 6 (severe diffuse) (Halbur et al., 1995). Lymphoid tissues were evaluated for lymphoid depletion and histiocytic replacement of follicles with scores ranging from 0 (none) to 3 (severe) (Opriessnig et al., 2004). Other tissues were evaluated for presence of inflammation ranging from 0 (none) to 3 (severe). Detection of PCV2 antigen was performed by PCV2 IHC using a rabbit polyclonal antiserum (Sorden et al., 1999). The amount of PCV2 antigen was scored by a pathologist blinded to treatment status. Scores ranged from 0 (no signal) to 3 (abundant, diffuse staining).

2.10. Bacteriology

Selected tissues and serum samples were submitted to the Iowa State University Veterinary Diagnostic Laboratory for bacterial isolation. *Erysipelothrix rhusiopathiae* IHC was done on selected slides as described (Opriessnig et al., 2010b).

2.11. Statistical analysis

For cytokine data analysis, JMP[®] software version 11.0.0 (SAS Institute, Cary, NC, USA) was used. Summary statistics were calculated for both groups to assess the overall quality of the data set including normality. Statistical analysis of the data was performed by using t-

tests. A *P*-value of less than 0.05 was set as the statistically significant level.

3. Results

3.1. Clinical observations

Clinical signs of disease were not observed in any of the PPV1-infected pigs through the time of scheduled necropsy at D42. All PCV2-infected pigs were clinically unremarkable until D28 when pig 124 was found in the morning with severe labored respiration with open-mouth breathing and cyanosis. Two hours later the pig was recumbent with a rectal temperature of 39.4 °C and was euthanized. By D49, pig 125 developed pasty-to-liquid diarrhea and by D50 pig 121 developed similar diarrhea and both pigs showed mild respiratory distress. Both pigs were treated with ceftiofur crystalline free acid (Excede[®] Zoetis) intramuscularly and enrofloxacin (Baytril 100, Bayer Animal Health) subcutaneously. Pig 121 had mild respiratory distress on D51. Over the following days, both pigs had rectal temperatures of approximately 38 °C. On D56, pig 121 was found in sternal recumbency with rapid breathing, cyanosis and abdominal distention and was subsequently euthanized. Between D64 to D67, pig 125 again developed fluid green-to-yellow diarrhea often containing undigested feed and was treated with ceftiofur crystalline free acid and enrofloxacin. Pig 125 was euthanized on D117 when the pig was 24 weeks old. At that time the pig was active and alert.

3.2. Anti-PCV2 and anti-PPV1 antibody levels

All PPV1 pigs were negative for anti-PCV2 and anti-PPV1 antibodies at arrival and all pigs remained negative for PCV2 antibodies. Anti-PPV1 IgG antibodies were first detected in all three PPV1 pigs at D6 (blocking percentage 31.3–42). PPV1 antibody levels continued to increase and reached approximately 100% by D15. The PPV1 antibody titers remained at that level until termination of the study at D49 (Fig. 2A). All three PPV1 pigs had HI titers of 16384 at D21 and D35.

All PCV2 pigs were negative for anti-PCV2 and anti-PPV1 antibodies at arrival and remained negative for PPV1 antibodies. Anti-PCV2 IgG antibodies were first detected at D49 in pig 125 by the *in-house* indirect ELISA (S/P ratio 0.43) and this pig remained positive at approximately the same antibody level until termination of the study at D117 (S/P ratio of 0.59) (Fig. 2B). The same pig was identified as positive for PCV2 antibodies by the commercial blocking ELISA at D56 (antibody titer of 677) with a similar trend for antibody levels until D117 (antibody titer 550). PCV2 antibodies were not detected in pigs 121 or 124.

3.3. Prevalence and amount of PCV2 and PPV1 DNA in serum samples

All PPV1 pigs were negative for PCV2 and PPV1 DNA in serum samples at arrival and the pigs remained negative for PCV2 DNA. PPV1 DNA was first detected on D3 in all three PPV1 pigs (Fig. 3) and all remained viremic until D28. PPV1 DNA was detected sporadically at low levels on D28, D35 and D42 and all three pigs were negative for PPV1 DNA in serum on D48 (Fig. 3).

Similarly, all PCV2 pigs were negative for PCV2 and PPV1 DNA in serum samples at arrival and the pigs remained negative for PPV1 DNA. PCV2 DNA was first detected on D3 in all three PCV2 pigs (Fig. 3). PCV2 pigs developed increasing PCV2 levels in serum approaching levels in the 10 log range by D14 with no decrease in PCV2 levels in pigs 121 and 124 until death. Pig 125, which had similarly high PCV2 levels compared to the other two pigs, started to have slightly decreased levels around D49 (9 logs), D56 (8 logs) and it finally reached 7 logs by D79 and remained at this level until D117 when it was euthanized (Fig. 3).

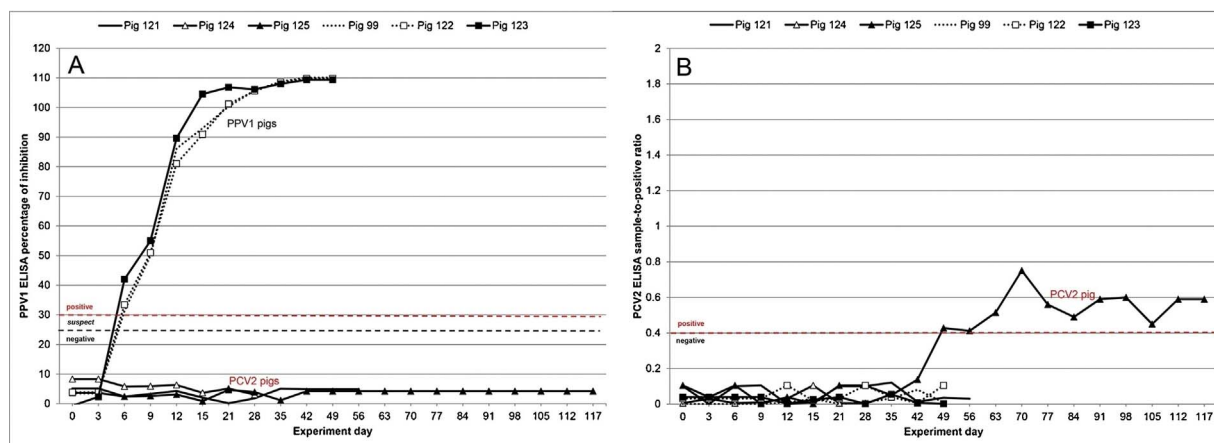


Fig. 2. Serological response to PPV1 (A) and PCV2 (B) over time using two commercial blocking ELISAs in pigs infected with PPV1 (99, 122 and 123) or PCV2 (121, 124 and 125) at D0. The red dashed line indicates the ELISA cut-off. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

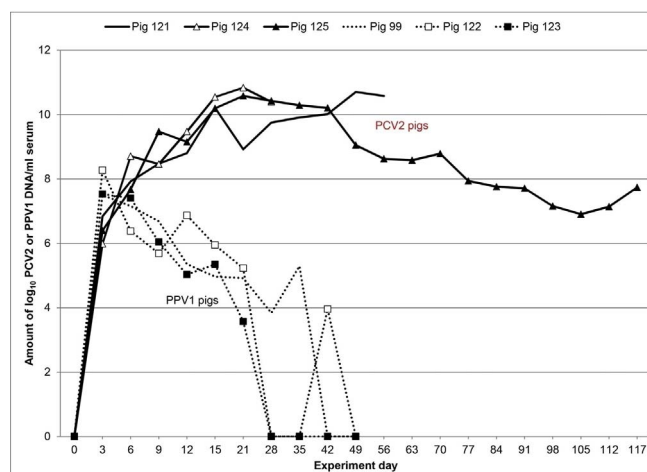


Fig. 3. Log₁₀ PCV2 genomic copies or PPV1 genomic copies per ml serum in pigs over time infected with PPV1 (99, 122 and 123) or PCV2 (121, 124 and 125) at D0.

3.4. Cytokine responses

The IFN- α , IL-8, IL-1 β and IFN γ cytokine responses are summarized in Fig. 4. In contrast to the PCV2 pigs, all three PPV1 pigs had a high INF- α response by D3 followed by a high IFN- γ response by D6. For TNF α , only one of the PPV1 infected pigs showed an increase in the TNF α levels at D6, whereas all the PCV2 infected pigs had elevated TNF α levels from D15 onwards (data not shown).

3.5. Gross lesions

The PPV1 pigs were necropsied at D42 when they were 11 weeks old. Pig 99 had a cystic left kidney, pig 122 had an inflamed bursal sac on the elbow joint and pig 123 had an inflamed bursal sac on the knee joint. There were no other remarkable macroscopic lesions in these pigs. All lymph nodes were of normal size and age appropriate. Among the PCV2 pigs, pig 124 (D28) had severe pulmonary edema, severe diffuse mottled tan-purple interstitial pneumonia, 30% cranioventral purple consolidation of the lung (Fig. 5A) and moderately enlarged lymph nodes. Pig 121 (D56) had approximately 1 l yellow fluid in the abdominal cavity and 1 l in the thoracic cavity and pericardium. Lungs were consolidated and tan to purple in color with moderate fibrinous pleuritis and tracheobronchial and mediastinal lymph nodes were markedly enlarged. There were multifocal 1–3 cm, round to rhomboid, red and slightly raised skin lesions most evident on the back and perineum (Fig. 5B). Pig 125 (D117) had diffuse severe enlargement of

tracheobronchial, renal, hepatic, mesenteric and gastric lymph nodes. Kidneys were tan, moderately enlarged with multifocal white areas of fibrosis (Fig. 5C). The liver had moderate to severe interstitial and lobular fibrosis (Fig. 5D). There were no remarkable lesions in lungs, small intestines and large intestines.

3.6. Microscopic lesions and PCV2 antigen in tissues

Microscopic lesions and PCV2 antigen levels are summarized in Table 1 and Fig. 6. Pig 124 (D28) had severe lymphoid depletion of lymph nodes and Peyer's patches with vasculitis (Fig. 6K). In lung sections, there was severe diffuse interstitial pneumonia with necrotizing and ulcerative bronchiolitis and interstitial and alveolar edema. In sections of liver there was moderate lymphohistiocytic hepatitis. In addition, there was severe granulomatous interstitial nephritis (Fig. 6D). Overall, the lesions in this pig were consistent with systemic PCVAD. Pig 121 (D56) had severe lymphoid depletion with histiocytic replacement of follicles, necrosis and hemorrhage in the lung with mild pulmonary edema, moderate lymphohistiocytic interstitial nephritis (Fig. 6E, H) and severe granulomatous enteritis associated with abundant PCV2 antigen (Fig. 6K). Due to the history of diarrhea and large amounts of PCV2 antigen in enteric sections, this pig was diagnosed with enteric PCVAD following systemic PCVAD. Pig 125 (D117) had mild lymphoid depletion and histiocytic replacement of lymphoid follicles (Fig. 6L, O), severe interstitial nephritis (Fig. 6F, I) and lymphohistiocytic myocarditis (Fig. 6D) and was diagnosed with chronic systemic PCVAD.

3.7. Bacteriology

There was no significant growth from lungs, spleen and serum from pigs 121 and 124 which was expected as both pigs had been treated with antimicrobials. *Erysipelothrix rhusiopathiae* IHC stains were negative.

4. Discussion

This report describes the clinical findings and PCV2 kinetics in three high health pigs infected with a PCV2a strain and compares and contrasts the findings with age-matched pigs from the same litter infected with PPV1. All three PCV2 pigs had a high and persistent PCV2 viremia without detectable PCV2 antibody response other than a weak reaction in one of the pigs after D49. In contrast, PPV1-infected pigs similarly became PPV1 viremic after infection and mounted a rapid and high immune response, which was correlated with virus clearance in these pigs by D42 after infection. All three PCV2 pigs developed clinical

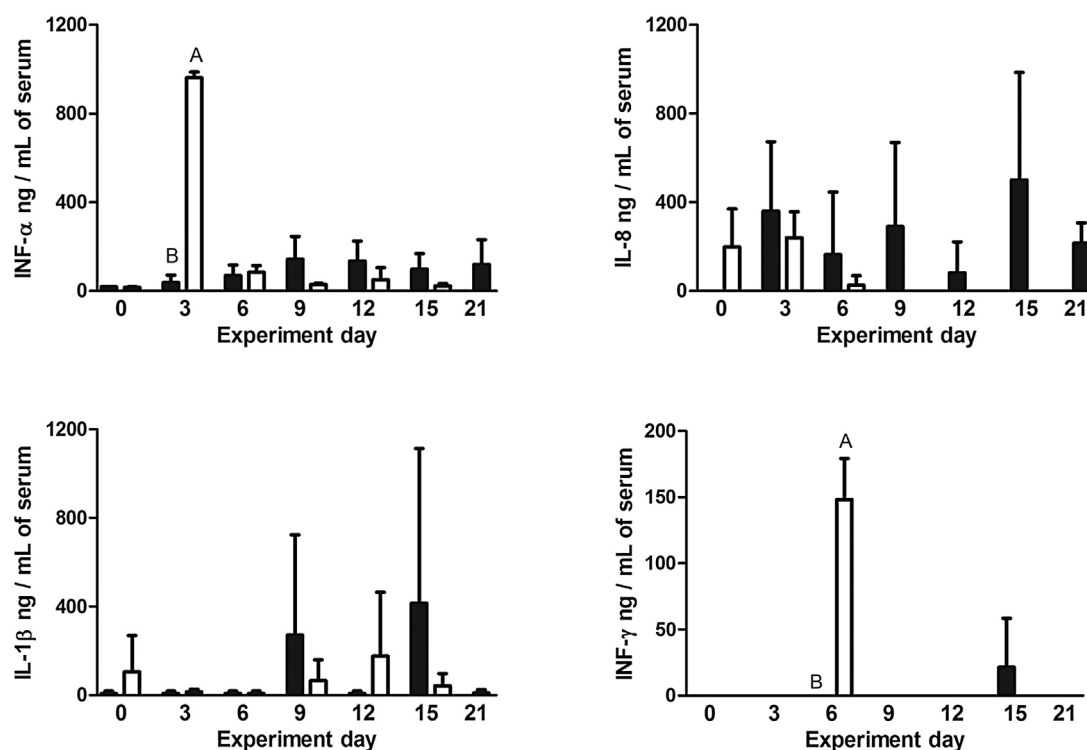


Fig. 4. Mean group cytokine responses including INF-α, IL-8, IL-1, and INF-γ in ng/mL serum measured at the day of inoculation (D0) and again at D3, D6, D9, D12, D15 and D21 in pigs infected with PPV1 (white bars) or PCV2 (black bars). Different superscripts (A,B) at a trial day indicate significant ($P < 0.05$) different group means.

disease at different times after inoculation and the disease course in two of the three pigs progressed to the point where euthanasia was required. The lesions in the PCV2 pigs were consistent with PCVAD lesions. No other agents were detected in any of the pigs, lesions characteristic of bacterial septicemia were not seen, and all pigs were only infected with the pathogen they were inoculated with (i.e. PCV2a or PPV1). Other factors that could have contributed to the development of PCVAD with PCV2a infection alone as described in this study could include a genetic predisposition of these pigs (all were from the same litter) and/or a higher susceptibility of CDCD pigs to infectious diseases in general.

It is well recognized that PCV2 infection can persist and that PCV2 DNA can be detected over extended periods of time in serum samples. Previously, conventionally raised (not CDCD) pigs remained PCV2 viremic for at least 140 days after PCV2 infection (Opriessnig et al., 2010a). In that study, IgM and IgG antibodies against PCV2 appeared between 14 and 21 days after infection and viral titers in serum decreased by several logs over the following weeks reaching 3–4 logs by D70. Some immunological differences can be attributed to difference between CDCD pigs versus conventional pigs. However, in other studies also using CDCD pigs, seroconversion to PCV2 was observed by D21 in some pigs (Opriessnig et al., 2014a, 2014b). In most experimental studies published in the literature, PCV2-infected pigs are euthanized at the anticipated peak of disease between D21 and D35 and data beyond that time are rare. In the present study, none of the pigs developed antibodies prior to D49 and the viral titers remained at 10 logs until D49, decreasing to 8 logs over the following weeks. Treatment with antimicrobials could also affect host response and the frequent antimicrobial treatment of the PCV2 pigs could have impacted their immune response.

Lymphoid lesions were severe in pig 124 (D28; abundant PCV2 antigen) and in pig 121 (D56; low-to-moderate amounts of PCV2 antigen) while lymphoid lesions were essentially resolved in pig 124 (D117) (Fig. 6M–O). Previous studies using experimental PCV2 infection have found that PCV2 associated lymphoid lesions are most severe between D14 and D21 and usually resolve by D35 (Hoogland et al.,

2006; Opriessnig et al., 2006a). The presence of severe lymphoid depletion at D56 is unusual and likely reflected by the uncontrolled persisting PCV2 infection.

Enteric PCVAD is an important differential diagnosis for granulomatous enteritis in pigs. In this study pigs 124 (D28) and 121 (D56) developed lymphohistiocytic enteritis associated with large amounts of PCV2 antigen in pig 121 (Fig. 6K). Shortly before euthanasia, this pig developed red raised skin lesions clinically indistinguishable from PDNS or acute bacterial septicemia. It is possible that the pig was immunocompromised and the integrity of its gut mucosal barrier was lost leading to secondary bacterial infection and acute septicemia.

Lymphohistiocytic interstitial nephritis is commonly seen with PCV2 infection (Opriessnig and Langohr, 2013). It has been suggested that renal lesions indicate chronicity of PCV2 infection with increasing prevalence after D35. However, because in previous studies these lesions were absent in experimental pigs by D69 and D140, eventual resolution of these lesions has been suggested (Opriessnig and Langohr, 2013). In this study, moderate-to-severe renal lesions (Fig. 6G–I) were present in all three PCV2 pigs and amount of PCV2 antigen in these lesions increased with time (Fig. 6D–F) and was highest in pig 125 (D117). Pig 125 had very mild lymphoid lesions highlighting that assessment of several organ systems may be needed to determine disease causality.

It is well recognized that PCV2 replicates in endothelial cells (Steiner et al., 2008) and is frequently associated with cardiovascular lesions in experimentally infected pigs (Opriessnig et al., 2006b) but also in field cases (Resendes and Segalés, 2015). Cardiovascular lesions were seen in all pigs and associated with the severe interlobular pulmonary edema and severe diffuse segmental to circumferential lymphohistiocytic and plasmacytic periarthritis and endarteritis in several organs in pigs 124 (D28) and 121 (D56) in this study. Diffuse distention of the pulmonary interlobular septa by edema and fibrinoid necrosis of the blood vessel wall has been described when PCV2b first entered the North American pig population and was attributed to differences between PCV2 strains (Carman et al., 2008). In this study, two of three

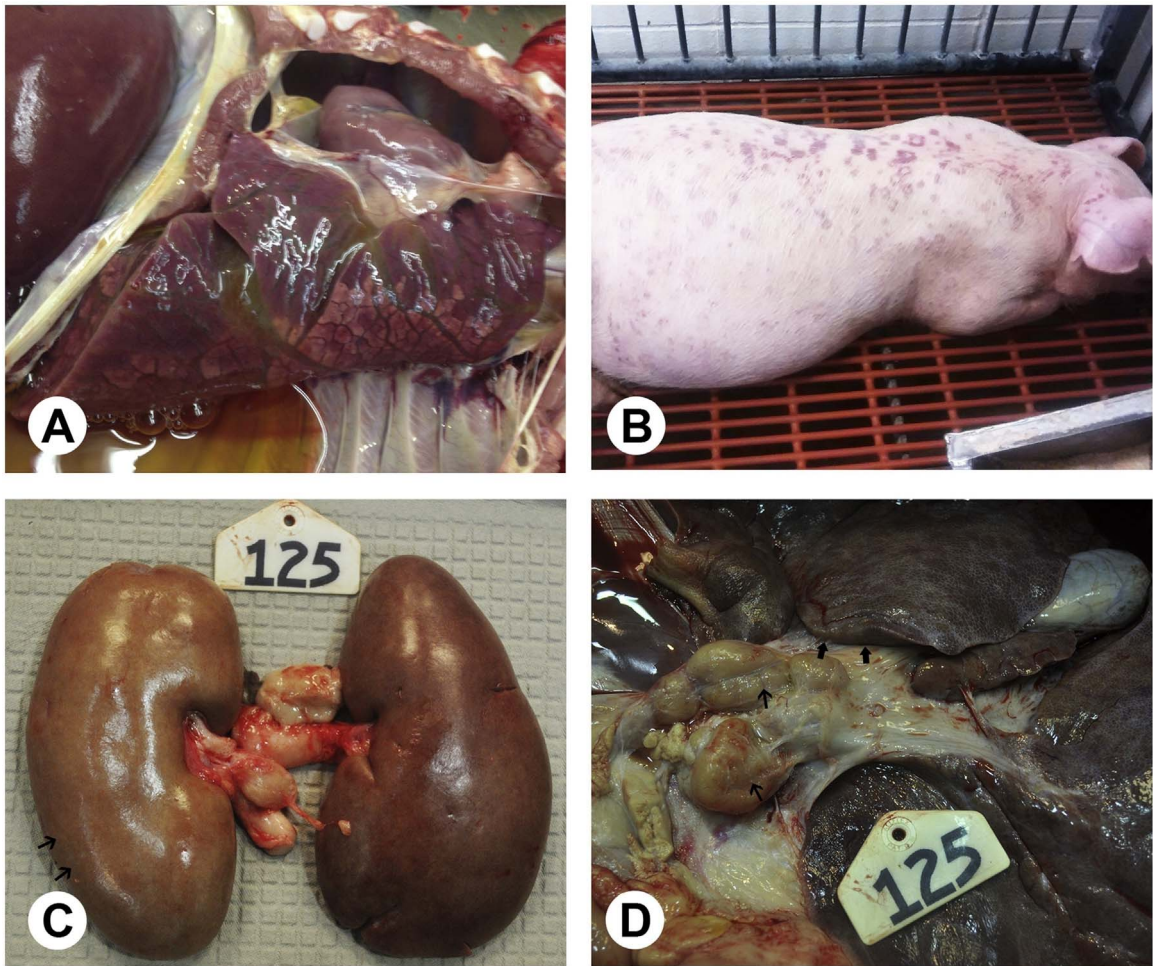


Fig. 5. Macroscopic lesions in the PCV2 infected pigs. **A.** Lung, pig 124, D28. There is multifocal severe dark-purple consolidation and moderate interlobular edema. **B.** Pig 121, D56. The pig is recumbent and there are multifocal 1–3 cm, round to rhomboid, red and slightly raised skin lesions on the back. **C.** Kidneys, pig 125, D117. There are multifocal small foci of fibrosis (arrows). **D.** Liver, hepatic and gastric lymph nodes. The liver surface has multifocal areas of fibrosis and the liver edges are rounded (bold arrows). Lymph nodes (normal arrows) are markedly enlarged. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

PCV2a infected pigs developed severe interlobular pulmonary edema. In fetuses, PCV2 replicates in myocardiocytes (Sanchez Jr et al., 2004) and the myocarditis is often associated with infiltration by lymphocytes, plasma cells, and macrophages of varying degrees (Opriessnig et al., 2006b). Severe chronic myocardial lesions were only present in pig 125 (D117) at 24 weeks of age but not in pigs 124 (D28) or 121 (D56) (Fig. 6A–C). This finding is unusual as pigs with PCV2 associated myocarditis are typically much younger and often the lesions are a result of a previous chronic intrauterine PCV2 infection (Mikami et al., 2005).

As the PPV1 pigs seroconverted early, the immune system in these pigs appeared intact and capable of mounting an immune response. PPV1 pigs had a significantly higher IFN- α response by D3 compared to

PCV2 pigs. IFN- α is usually produced by virus infected host cells and provides resistance to viral replication while increasing MHC-I expression and antigen presentation and activating NK cells. It is thought that IFN- γ secreting cells develop during the adaptive response to PCV2 and probably contribute to viral clearance in infected pigs (Fort et al., 2009). In this study IFN- γ , while present in PPV1 pigs in high levels at D6, was not detected in the PCV2 pigs. This could explain the high PCV2 virus loads in these pigs over the course of the study.

Both IL-8 and IL-1 β are secreted by activated macrophages. IL-8 induces a local inflammatory response including increased angiogenesis and IL-1 β activates lymphocytes and induces local tissue destruction. PCV2 has the ability to induce strong IL-1 β and IL-8 responses in peripheral blood mononuclear cells (PBMCs) of both naïve and PCV2-

Table 1
Microscopic lesions in pigs infected with PCV2 28, 56 or 117 days previously and presence and amount PCV2 antigen associated with the lesions as determined by immunohistochemistry. Data presented as severity score ranging from 0 = normal; 1 = mild; 2 = moderate to 3 = severe (IHC score ranging from negative (–) to abundant (+++)).

	Lymph nodes LD and HR of follicles	Lung Interlobular edema	Peri-bronchiolar fibrosis	Liver LH hepatitis	Kidney LH-G interstitial and tubular nephritis	Heart LH myocarditis	Ileum LD of Peyer's patches	LH enteritis
Pig 124 (D28)	3 (+++)	3 (+++)	2 (+++)	1 (++)	3 (++)	0 (0)	3 (+++)	1 (+)
Pig 121 (D56)	3 (+/+ +) NEC	2 (++)	2 (+) NEC	1 (++)	2 (+++)	0 (0)	1 (+++)	3 (+++)
Pig 125 (D117)	1 (-/+) ^a	1 (–)	0 (–)	1 (–)	3 (+++)	3 (+)	0 (–)	0 (–)

Abbreviations used: LH = lymphohistiocytic; NEC = multifocal necrosis; G = granulomatous; HR = histiocytic replacement; LD = lymphoid depletion.

^a Spleen: focal severe histiocytic replacement of follicles.

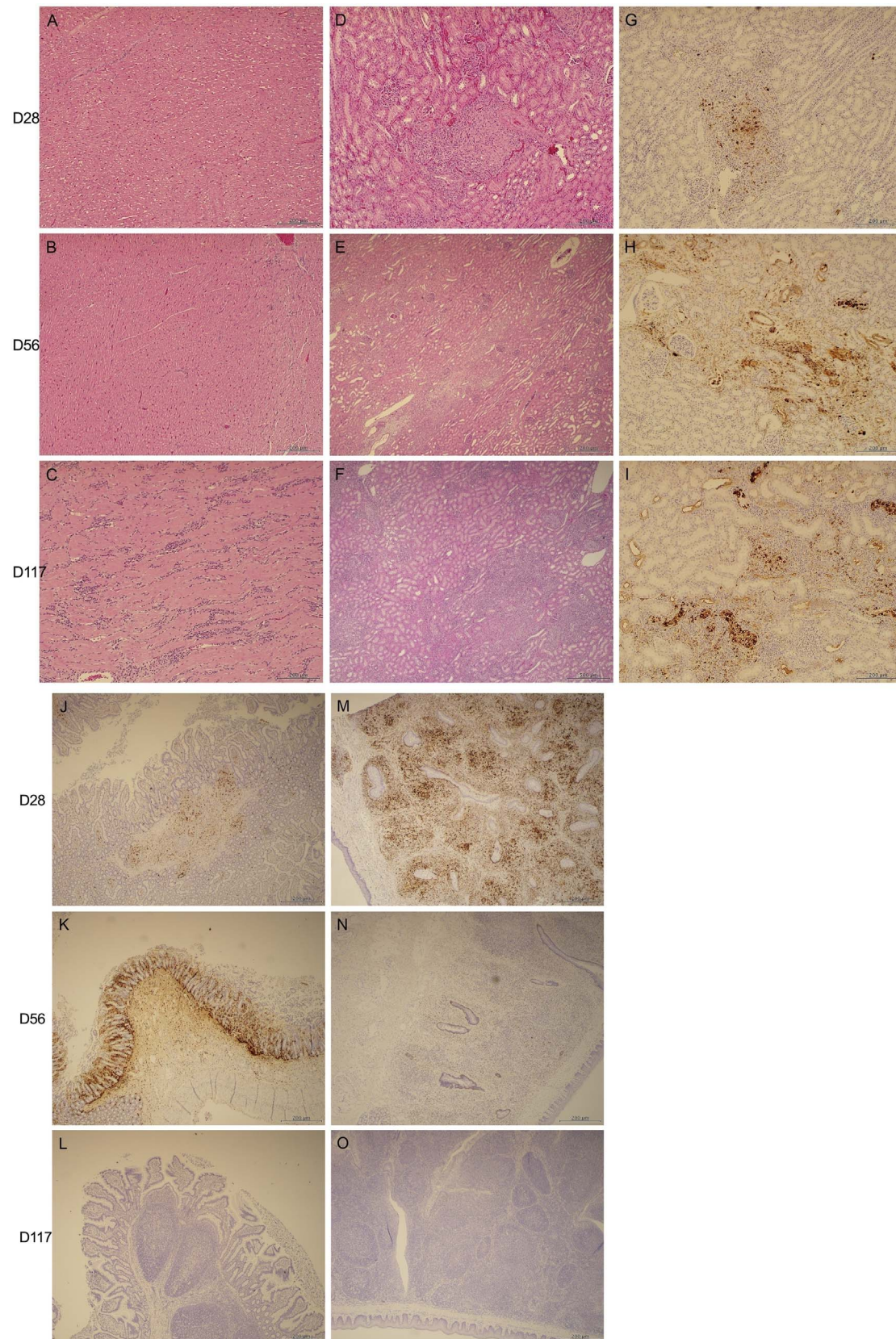


Fig. 6. Microscopic lesions in the PCV2 infected pigs at D28 (top row), D56 (middle row) or D117 (bottom row) post infection. **A-C.** Heart, HE. **A, B:** Normal. **C:** Moderate diffuse lymphohistiocytic myocarditis. **D-F.** Kidney, HE. Mild-to-moderate, multifocal lymphohistiocytic tubulointerstitial nephritis. **G-I.** Kidney, PCV2 IHC. Moderate to high amount of PCV2 antigen associated with areas of inflammation and renal tubular necrosis. **J-L:** Ileum, PCV2 IHC. There is no (**L**), low (**J**) to high (**K**) amounts of PCV2 antigen present in the lamina propria and submucosa of the intestinal sections. **M-O.** Tonsil, PCV2 IHC. **M:** Moderate lymphoid depletion associated with large amounts of PCV2 antigen. **N:** Severe lymphoid depletion with only a few cells staining positive for PCV2 antigen. **O:** Normal tonsil negative for PCV2 antigen.

infected pigs (Darwich et al., 2003). In this study, an IL-8 response was seen in PCV2 pigs from D9 through D21 but not in PPV1 pigs. PPV1 or PCV2 infected pigs had spikes of IL-1 β starting at D9 which is typical of chronic inflammation as commonly seen in PCVAD.

TNF α is a pro-inflammatory cytokine associated with infections, and is known to promote loss of epithelial barrier integrity, edema and cachexia (Capaldo and Nusrat, 2009; Mullin and Snock, 1990). In this study only pig 99 in the PPV2 infected group showed a transient increase in TNF α levels on D6 through D9 while TNF α was not detected in any of the other PPV1 pigs. All PCV2 infected pigs; however, consistently showed elevated levels of TNF α starting at D15 which remained elevated. The association between elevated TNF α levels in PCV2 infected pigs and progressive weight loss has been documented earlier (Kim et al., 2006; Kreikemeier et al., 2015). Interestingly, in this study, where other infections have been ruled out, PCV2 alone was sufficient to increase the systemic TNF α levels. This observation may warrant further analysis of TNF α as a marker in diagnosis of PCVAD.

5. Conclusions

The differences in responses of CDCD pigs to two common single-stranded DNA viruses, PCV2 and PPV1, were compared and contrasted. Under the study conditions, clinical signs and lesions consistent with PCVAD were reproduced in pigs infected with a PCV2a isolate from 2000. This study highlights the unique immune response to PCV2 in some pigs and clearly shows that PCV2 can induce systemic cytokine changes, even in the absence of other known pathogens. This pathogenesis could be exacerbated by concurrent infection with other pathogens. This study reiterates the importance of controlling PCV2 infection in commercial pig herds.

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Conflict of interest statement

The authors declare no financial and personal relationships with other people or organizations that could inappropriately influence this work.

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References

Capaldo, C.T., Nusrat, A., 2009. Cytokine regulation of tight junctions. *Biochim. Biophys. Acta* 1788, 864–871.

Carman, S., Cai, H.Y., DeLay, J., Youssef, S.A., McEwen, B.J., Gagnon, C.A., Tremblay, D., Hazlett, M., Lusi, P., Fairles, J., Alexander, H.S., van, D.T., 2008. The emergence of a new strain of porcine circovirus-2 in Ontario and Quebec swine and its association with severe porcine circovirus associated disease-2004-2006. *Can. J. Vet. Res.* 72, 259–268.

Cheung, A.K., Wu, G., Wang, D., Bayles, D.O., Lager, K.M., Vincent, A.L., 2010. Identification and molecular cloning of a novel porcine parvovirus. *Arch. Virol.* 155, 801–806.

Darwich, L., Balasch, M., Plana-Dúran, J., Segalés, J., Domingo, M., Mateu, E., 2003. Cytokine profiles of peripheral blood mononuclear cells from pigs with postweaning multisystemic wasting syndrome in response to mitogen, superantigen or recall viral antigens. *J. Gen. Virol.* 84, 3453–3457.

Dunne, H.W., Gobble, J.C., Hokanson, J.R., Kradel, D.D., Bubash, G.R., 1965. Porcine reproductive failure associated with a newly defined SMEDI group of picornaviruses. *Am. J. Vet. Res.* 26, 1284–1290.

Fenaux, M., Halbur, P.G., Gill, M., Toth, T.E., Meng, X.J., 2000. Genetic characterization of type 2 porcine circovirus (PCV-2) from pigs with postweaning multisystemic wasting syndrome in different geographic regions of North America and development of a differential PCR-restriction fragment length polymorphism assay to detect and differentiate between infections with PCV-1 and PCV-2. *J. Clin. Micro.* 38, 2494–2503.

Fenaux, M., Opriessnig, T., Halbur, P.G., Meng, X.J., 2003. Immunogenicity and pathogenicity of chimeric infectious DNA clones of pathogenic porcine circovirus type 2 (PCV2) and nonpathogenic PCV1 in weanling pigs. *J. Virol.* 77, 11232–11243.

Fort, M., Fernandes, L.T., Nofrarias, M., Diaz, I., Sibila, M., Pujols, J., Mateu, E., Segales, J., 2009. Development of cell-mediated immunity to porcine circovirus type 2 (PCV2) in caesarean-derived, colostrum-deprived piglets. *Vet. Immunol. Immunopathol.* 129, 101–107.

Gerber, P.F., Johnson, J., Shen, H., Striegel, D., Xiao, C.T., Halbur, P.G., Opriessnig, T., 2013. Association of concurrent porcine circovirus (PCV) 2a and 2b infection with PCV associated disease in vaccinated pigs. *Res. Vet. Sci.* 95, 775–781.

Halbur, P.G., Paul, P.S., Frey, M.L., Landgraf, J., Eernisse, K., Meng, X.J., Lum, M.A., Andrews, J.J., Rathje, J.A., 1995. Comparison of the pathogenicity of two US porcine reproductive and respiratory syndrome virus isolates with that of the Lelystad virus. *Vet. Pathol.* 32, 648–660.

Harding, J.C., 2007. Status of Porcine circovirus diseases in western Canada. *Can. Vet. J.* 48, 267–268.

Harms, P.A., Halbur, P.G., Sorden, S.D., 2002. Three cases of porcine respiratory disease complex associated with porcine circovirus type 2 infection. *J. Swine Health Prod.* 10, 27–30.

Hijikata, M., Abe, K., Win, K.M., Shimizu, Y.K., Keicho, N., Yoshikura, H., 2001. Identification of new parvovirus DNA sequence in swine sera from Myanmar. *Jpn. J. Infect. Dis.* 54, 244–245.

Hoogland, M.J., Opriessnig, T., Halbur, P.G., 2006. Effects of adjuvants on porcine circovirus type 2-associated lesions. *J. Swine Health Prod.* 14, 133–139.

Jensen, T.K., Vigre, H., Svensmark, B., Bille-Hansen, V., 2006. Distinction between porcine circovirus type 2 enteritis and porcine proliferative enteropathy caused by Lawsonia intracellularis. *J. Comp. Pathol.* 135, 176–182.

Kim, J., Chung, H.K., Chae, C., 2003. Association of porcine circovirus 2 with porcine respiratory disease complex. *Vet. J.* 166, 251–256.

Kim, J., Ha, Y., Chae, C., 2006. Potentiation of porcine circovirus 2-induced postweaning multisystemic wasting syndrome by porcine parvovirus is associated with excessive production of tumor necrosis factor- α . *Vet. Pathol.* 43, 718–725.

Kreikemeier, C.A., Engle, T.B., Lucot, K.L., Kachman, S.D., Burke, T.E., Ciobanu, D.C., 2015. Genome-wide analysis of TNF- α response in pigs challenged with porcine circovirus 2b. *Anim. Genet.* 46, 205–208.

Kresse, J.I., Taylor, W.D., Stewart, W.W., Eernisse, K.A., 1985. Parvovirus infection in pigs with necrotic and vesicle-like lesions. *Vet. Microbiol.* 10, 525–531.

Ladinig, A., Lunney, J.K., Souza, C.J., Ashley, C., Plastow, G., Harding, J.C., 2014. Cytokine profiles in pregnant gilts experimentally infected with porcine reproductive and respiratory syndrome virus and relationships with viral load and fetal outcome. *Vet. Res.* 45, 113.

Lager, K.M., Mengeling, W.L., 1994. Porcine parvovirus associated with cutaneous lesions in piglets. *J. Vet. Diagn. Invest.* 6, 357–359.

Lau, S.K., Woo, P.C., Tse, H., Fu, C.T., Au, W.K., Chen, X.C., Tsoi, H.W., Tsang, T.H., Chan, J.S., Tsang, D.N., Li, K.S., Tse, C.W., Ng, T.K., Tsang, O.T., Zheng, B.J., Tam, S., Chan, K.H., Zhou, B., Yuen, K.Y., 2008. Identification of novel porcine and bovine parvoviruses closely related to human parvovirus 4. *J. Gen. Virol.* 89, 1840–1848.

Lawson, S., Lunney, J., Zuckermann, F., Osorio, F., Nelson, E., Welbon, C., Clement, T., Fang, Y., Wong, S., Kulas, K., Christopher-Hennings, J., 2010. Development of an 8-plex Luminex assay to detect swine cytokines for vaccine development: assessment of immunity after porcine reproductive and respiratory syndrome virus (PRRSV) vaccination. *Vaccine* 28, 5356–5364.

Mengeling, W.L., Ridpath, J.F., Vorwald, A.C., 1988. Size and antigenic comparisons among the structural proteins of selected autonomous parvoviruses. *J. Gen. Virol.* 69 (Pt 4), 825–837.

Mengeling, W.L., Lager, K.M., Zimmerman, J.K., Samarikermani, N., Beran, G.W., 1991. A current assessment of the role of porcine parvovirus as a cause of fetal porcine death. *J. Vet. Diagn. Invest.* 3, 33–35.

Mengeling, W.L., 1979. Prenatal infection following maternal exposure to porcine parvovirus on either the seventh or fourteenth day of gestation. *Can. J. Comp. Med.* 43, 106–109.

Mikami, O., Nakajima, H., Kawashima, K., Yoshii, M., Nakajima, Y., 2005. Nonsuppurative myocarditis caused by porcine circovirus type 2 in a weak-born piglet. *J. Vet. Med. Sci.* 67, 735–738.

Mullin, J.M., Snock, K.V., 1990. Effect of tumor necrosis factor on epithelial tight junctions and transepithelial permeability. *Cancer Res.* 50, 2172–2176.

Nawagitgul, P., Harms, P.A., Morozov, I., Thacker, B.J., Sorden, S.D., Lekcharoensuk, C., Paul, P.S., 2002. Modified indirect porcine circovirus (PCV) type 2-based and recombinant capsid protein (ORF2)-based enzyme-linked immunosorbent assays for detection of antibodies to PCV. *Clin. Diagn. Lab. Immunol.* 9, 33–40.

Ni, J., Qiao, C., Han, X., Han, T., Kang, W., Zi, Z., Cao, Z., Zhai, X., Cai, X., 2014. Identification and genomic characterization of a novel porcine parvovirus (PPV6) in China. *Virol. J.* 11, 203.

Opriessnig, T., Langohr, I., 2013. Current state of knowledge on porcine circovirus type 2-associated lesions. *Vet. Pathol.* 50, 23–38.

Opriessnig, T., Yu, S., Gallup, J.M., Evans, R.B., Fenaux, M., Pallarés, F.J., Thacker, E.L., Brockus, C.W., Ackermann, M.R., Thomas, P., Meng, X.J., Halbur, P.G., 2003. Effect

- of vaccination with selective bacterins on conventional pigs infected with type 2 porcine circovirus. *Vet. Pathol.* 40, 521–529.
- Opriessnig, T., Thacker, E.L., Yu, S., Fenaux, M., Meng, X.J., Halbur, P.G., 2004. Experimental reproduction of postweaning multisystemic wasting syndrome in pigs by dual infection with *Mycoplasma hyopneumoniae* and porcine circovirus type 2. *Vet. Pathol.* 41, 624–640.
- Opriessnig, T., Fenaux, M., Thomas, P., Hoogland, M.J., Rothschild, M.F., Meng, X.J., Halbur, P.G., 2006a. Evidence of breed-dependent differences in susceptibility to porcine circovirus type-2-associated disease and lesions. *Vet. Pathol.* 43, 281–293.
- Opriessnig, T., Janke, B.H., Halbur, P.G., 2006b. Cardiovascular lesions in pigs naturally or experimentally infected with porcine circovirus type 2. *J. Comp. Pathol.* 134, 105–110.
- Opriessnig, T., Meng, X.J., Halbur, P.G., 2007. Porcine circovirus type 2 associated disease: update on current terminology, clinical manifestations, pathogenesis, diagnosis, and intervention strategies. *J. Vet. Diagn. Invest.* 19, 591–615.
- Opriessnig, T., Ramamoorthy, S., Madson, D.M., Patterson, A.R., Pal, N., Carman, S., Meng, X.J., Halbur, P.G., 2008. Differences in virulence among porcine circovirus type 2 isolates are unrelated to cluster type 2a or 2b and prior infection provides heterologous protection. *J. Gen. Virol.* 89, 2482–2491.
- Opriessnig, T., Prickett, J., Madson, D.M., Shen, H.G., Juhan, N.M., Pogranichniy, R., Meng, X.J., Halbur, P.G., 2010a. Porcine circovirus type 2 (PCV2)-infection and re-inoculation with homologous or heterologous strains: virological, serological, pathological and clinical effects in growing pigs. *Vet. Res.* 41, 31.
- Opriessnig, T., Bender, J.S., Halbur, P.G., 2010b. Development and validation of an immunohistochemical method for rapid diagnosis of swine erysipelas in formalin-fixed, paraffin-embedded tissue samples. *J. Vet. Diagn. Invest.* 22, 86–90.
- Opriessnig, T., Madson, D.M., Roof, M., Layton, S.M., Ramamoorthy, S., Meng, X.J., Halbur, P.G., 2011a. Experimental reproduction of porcine circovirus type 2 (PCV2)-associated enteritis in pigs infected with PCV2 alone or concurrently with *Lawsonia intracellularis* or *Salmonella typhimurium*. *J. Comp. Pathol.* 145, 261–270.
- Opriessnig, T., Shen, H.G., Pal, N., Ramamoorthy, S., Halbur, P.G., 2011b. A live-attenuated chimeric PCV2 vaccine is transmitted to contact pigs but is not upregulated by concurrent infection with PPV and PRRSV and is efficacious in a PCV2a-PRRSV-PPV challenge model. *Clin. Vaccine Immunol.* 18, 1261–1268.
- Opriessnig, T., Gerber, P.F., Xiao, C.T., Mogler, M., Halbur, P.G., 2014a. A commercial vaccine based on PCV2a and an experimental vaccine based on a variant mPCV2b are both effective in protecting pigs against challenge with a 2013 U.S. variant mPCV2b strain. *Vaccine* 32, 230–237.
- Opriessnig, T., Xiao, C.T., Gerber, P.F., Halbur, P.G., Matzinger, S.R., Meng, X.J., 2014b. Mutant U.S. strain of porcine circovirus type 2 (mPCV2) exhibits similar virulence to the classical PCV2a and PCV2b strains in caesarian-derived colostrum-deprived pigs. *J. Gen. Virol.* 95, 2495–2503.
- Palinski, R.M., Mitra, N., Hause, B.M., 2016. Discovery of a novel Parvovirinae virus, porcine parvovirus 7, by metagenomic sequencing of porcine rectal swabs. *Virus Genes* 52, 564–567.
- Patterson, A.R., Opriessnig, T., 2010. Epidemiology and horizontal transmission of porcine circovirus type 2 (PCV2). *Anim. Health Res. Rev.* 11, 217–234.
- Resendes, A.R., Segalés, J., 2015. Characterization of vascular lesions in pigs affected by porcine circovirus type 2-systemic disease. *Vet. Pathol.* 52, 497–504.
- Rosell, C., Segalés, J., Plana-Durán, J., Balasch, M., Rodríguez-Arriola, G.M., Kennedy, S., Allan, G.M., McNeilly, F., Latimer, K.S., Domingo, M., 1999. Pathological, immunohistochemical, and in-situ hybridization studies of natural cases of postweaning multisystemic wasting syndrome (PMWS) in pigs. *J. Comp. Pathol.* 120, 59–78.
- Sanchez Jr., R.E., Meerts, P., Nauwynck, H.J., Ellis, J.A., Pensaert, M.B., 2004. Characteristics of porcine circovirus-2 replication in lymphoid organs of pigs inoculated in late gestation or postnatally and possible relation to clinical and pathological outcome of infection. *J. Vet. Diagn. Invest.* 16, 175–185.
- Sorden, S.D., Harms, P.A., Nawagitgul, P., Cavanaugh, D., Paul, P.S., 1999. Development of a polyclonal-antibody-based immunohistochemical method for the detection of type 2 porcine circovirus in formalin-fixed, paraffin-embedded tissue. *J. Vet. Diagn. Invest.* 11, 528–530.
- Steiner, E., Balmelli, C., Herrmann, B., Summerfield, A., McCullough, K., 2008. Porcine circovirus type 2 displays pluripotency in cell targeting. *Virology* 378, 311–322.
- Xiao, C.T., Halbur, P., Opriessnig, T., 2013. Complete genome sequence of a novel porcine parvovirus (PPV) provisionally designated PPV5. *Genome Announc.* 1, e00021–12.
- Xiao, C.T., Halbur, P.G., Opriessnig, T., 2015. Global molecular genetic analysis of porcine circovirus type 2 (PCV2) sequences confirms the presence of four main PCV2 genotypes and reveals a rapid increase of PCV2d. *J. Gen. Virol.* 96, 1830–1841.